ISOLATION AND CHARACTERIZATION OF THE AMORPHOUS INCLUSIONS ASSOCIATED WITH PEPPER MOTTLE AND PAPAYA RINGSPOT VIRUSES

BY

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Maria V. G. de Mejia

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Viruses in the potyvirus group stimulate the formation of inclusion bodies in infected cells of their hosts. The cytoplasmic cylindrical inclusions (CI) associated with all potyvirus infections consist of nonstructural protein monomers with a molecular weight (Mr) of 67-70 X 10^3. Nuclear inclusions (NI) induced by tobacco etch virus consist of two distinct protein monomers with Mr of 49 and 54 X 10^3.

Most potyviruses are transmitted by aphids. This transmission requires the assistance of helper component (HC), a soluble protein with Mr of 52-58 X 10^3. All four nonstructural proteins (CI, two NI and HC) have been identified as virus-specified proteins.

Pepper mottle virus (PeMV) and the watermelon mosaic virus-1 strain of papaya ringspot virus (PRSV-W) induce the formation of a second type of cytoplasmic inclusion in addition to CI. Infections with these two potyviruses reveal conspicuous aggregates of amorphous, electron dense material that is also detectable by light microscopy. The staining reaction of these amorphous inclusions (AI) suggests protein and nucleic acid constituents, but the nature of these constituents and their biological significance are not known. The primary purpose of this study was to characterize the proteins associated with these AI.

The AIs were isolated using a combination of Triton X-100 clarification and low speed centrifugations through sucrose cushions. Sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) of the SDS dissociated AI revealed a single constituent protein with a Mr of 51 X 10^3. SDS-PAGE purified AI proteins were immunogenic and serologically related to HC protein, but distinct from host, capsid, CI, and NI proteins. The AI antisera reacted specifically with AI *in situ* in immunofluorescence tests.

In vitro translation products of PeMV and PRSV-W RNAs were compared with AI proteins using SDS-PAGE, immunoprecipitation and peptide mapping. The major translation products of PeMV and PRSV-W RNAs were immunoprecipitated by AI antisera and gave similar proteolytic peptides to those of AI proteins.

This study provides direct evidence for the viral origin of AI proteins, which represent another nonstructural protein identified for

the potyviral genome. The serological relationships of HC and AI proteins is the first correlation of a biologically functional protein with an inclusion-related protein in potyviral infections.

CHAPTER 1

INTRODUCTION

Potyviruses are filamentous plant viruses with a diameter of 12 nm and range in length from about 680 to 900 nm (Fenner, 1976). The viral genome consists of a single stranded nonsegmented RNA, sedimenting around 39S on sucrose density gradients, and with an estimated molecular weight (Mr) of 3.0-3.5 X 10^6 (Hari et al., 1979; Hill and Benner, 1976; Hinostroza-Orihuela, 1975; Pring and Langenberg, 1972; Vance and Beachy, 1984). The RNA has a small protein covalently attached to the 5' terminal nucleotide and a polyadenylate sequence at the 3' terminus (Han et al., 1979; Hari, 1981; Vance and Beachy, 1984). The capsid consists of protein monomers with an estimated Mr of 30,000-36,000 (30-36k) (Hiebert and McDonald, 1973; 1976).

One interesting and well established phenomenon associated with potyviral infections is the synthesis of large amounts of nonstructural proteins. These proteins are aggregated in host cells to form distinct morphological structures known as inclusions. Cylindrical inclusions (CI) are observed in the cytoplasm of all potyvirus-infected plants (Edwardson, 1974). The CIs consist of a protein monomer with a Mr of 67-70k (Hiebert and McDonald, 1973). Inclusions in the nucleus have been observed for only a limited number of potyviruses (Edwardson, 1974; Christie and Edwardson, 1977). The nuclear inclusions (NI) associated with tobacco etch virus (TEV) infections are composed of two distinct protein monomers with Mr 49k and 54k (Knuhtsen et al., 1974). These

inclusions are virus-specific and serologically unrelated to the capsid protein and to host protein (Purcifull et al., 1973; Knuhtsen et al., 1974). The two NI proteins and the CI protein have been identified as virus-coded nonstructural proteins on the basis of in vitro translation studies of TEV and pepper mottle virus (PeMV) (Dougherty and Hiebert, 1980b). In a series of recently published papers (Hellman et al., 1983; Thornbury and Pirone, 1983) the helper component (HC) required for aphid transmission of potyviruses has been identified as a nonstructural protein of tobacco vein mottling virus (TVMV)-RNA, unrelated to cylindrical or TEV-NI proteins.

Potyviruses may induce other types of cytoplasmic inclusions in addition to CI. Edwardson (1974) reported large, irregular-shaped inclusions in the cytoplasm of cells from plants infected with PeMV. Irregular-shaped, amorphous inclusions (AI) were also reported by Edwardson (1974) and Martelli and Russo (1976) in tissues infected with papaya ringspot virus type W (PRSV-W), previously known as watermelon mosaic virus-1 (Purcifull et al., in press). These AIs have been observed by light microscopy and in ultrathin sections by electron microscopy but studies to date leave unanswered many questions regarding the nature and biological properties of the potyviral infection associated inclusions. The purpose of this study was to resolve the properties of these inclusions by isolating the AIs and characterizing the proteins associated with them.

CHAPTER 2

ISOLATION OF AMORPHOUS INCLUSIONS INDUCED BY PEPPER MOTTLE VIRUS AND PAPAYA RINGSPOT VIRUS (WATERMELON MOSAIC VIRUS-1 STRAIN). PURIFICATION AND IMMUNOLOGICAL ANALYSES OF AMORPHOUS INCLUSION CONSTITUENT PROTEIN

Introduction

The occurrence of large, cytoplasmic amorphous inclusions (AI), which are also known as irregular inclusions (Christie and Edwardson, 1977), in tissues infected with pepper mottle virus (PeMV) and a watermelon mosaic virus-1 strain of papaya ringspot virus (PRSV-W) (Purcifull et al., in press), has been reported by Edwardson (1974) and Martelli and Russo (1976). Reports regarding the nature of the AI are limited to in situ studies. Martelli and Russo (1976), who studied the cytochemical staining reaction and enzyme digestion properties of AI induced by PRSV-W, provided evidence that the AI were composed mainly of protein and accompanying RNA. They state that the AI might be accumulations of excess virus coat protein but, since the AI contain the basic elements required for virus assembly, they did not rule out the possibility that the AI could represent sites of virus assembly and/or multiplication.

I have investigated the nature of AI by isolating and studying them in vitro. In this chapter a purification scheme for the AI associated with PeMV and PRSV-W and a partial characterization of these inclusions will be presented.

Materials and Methods

Virus source and propagation.--PRSV-W Florida isolate (Purcifull and Hiebert, 1979) was propagated in zucchini squash Cucurbita pepo var. Chefini. The PeMV isolate (Purcifull et al., 1975) was maintained in Nicotiana tabacum L. var. NN. Plants held at 20-30° were manually inoculated and the virus isolates were periodically transferred from plant to plant by sap inoculation. Plants were harvested between four and six weeks after inoculation.

Isolation of amorphous inclusions and purification of amorphous inclusions constituent protein.--Systemically infected tissue was blended for 2 min at high speed in a Waring blender, using 1 ml of an ice cold solution of extraction buffer (EB), which consisted of 100 mM Tris, pH 7.5, and 0.5% sodium sulfite, per gram of tissue. The tissue homogenate was filtered through two layers of cheesecloth and centrifuged at 4000 g for 5 min through a 30 ml cushion of 20% sucrose in EB. The pellets were resuspended in cold EB. Triton X-100 was added to a final concentration of 5% and the resulting mixture was stirred for 1 hr at 4°. The preparation was centrifuged at 4000 g for 5 min through a 15 ml cushion of 40% sucrose in EB. The pellets were resuspended in cold EB. Triton X-100 was added to a final concentration of 5%. The resulting mixture was stirred for 15 min at 4°. This procedure was repeated for a total of three successive 40% sucrose cushion centrifugations or until no more green colored material was evident in the pellets. The final pellets were resuspended in a small volume of 100 mM Tris pH 7.5 and stored at -20°. Hereafter this preparation will be referred as semipurified AI.

Purification of the AI constituent protein was obtained with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using the

Laemmli (1970) discontinuous buffer system. A linear 7.5 to 15% gel gradient was used in a slab gel apparatus (3 X 140 X 150 mm). The semipurified AI preparation was dissociated by mixing with an equal volume of a solution containing 100 mM Tris-HCl, pH 6.8, 2.5% SDS, 5% 2-mercaptoethanol (ME) and 5% sucrose, and heated at 90° for 3-5 min. The sample was then layered on top of the gel and electrophoresed for 14 hr at a constant voltage of 80. The AI protein band was detected in the gel by labeling a portion of the AI protein with dansyl chloride prior to electrophoresis (Talbot and Yphantis, 1971). The gel band, containing the AI protein, was excised and crushed with a mortar and pestle. Ten volumes of water were added to the crushed gel. This material was frozen at -20° for a few hours and then incubated at room temperature for 18 hr. The material was centrifuged at 1000 g for 5 min. The pellet was reextracted with an equal volume of water and centrifuged at 1000 g for 5 min. The supernatants were combined and filtered through a Millipore membrane (HA, 0.45 μm), and then water was removed by freeze-drying. The residue was resuspended in 2 ml of deionized water and dialyzed for 8 hr against deionized water. Yields were estimated by spectrophotometry as A_{280} and the dialyzed protein was stored at -20°.

Antisera production and immunodiffusion tests.--Antisera to the AI proteins were prepared in New Zealand white rabbits acording to Hiebert et al. (in press a). Freeze-dried antigen (c. 1 mg) in 1 ml of deionized water was homogenized with 1 ml of Freund's complete adjuvant. Part of this emulsion (0.15 ml) was injected into the rabbit's footpads, while the rest of the emulsion was administered intramuscularly into the hind legs. This injection procedure was repeated two times, using incomplete Freund's adjuvant. For the PeMV-AI protein, the rabbit received the

second and third injection fifteen and thirty days after the first injection. The rabbit receiving PRSV-W AI protein was injected at eighteen day intervals. The rabbits were bled at weekly intervals after the final injection. The PeMV-AI and PRSV-W AI antisera used were from rabbit number 1040 (collected April 7, 1983) and rabbit number 1042 (collected April 7, 1983), respectively.

SDS immunodiffusion tests were conducted as described by Purcifull and Batchelor (1977). Purified AI proteins were dissociated in 1% SDS. Crude, SDS-treated antigens were prepared from freshly harvested leaves of infected and noninfected tobacco and zucchini squash plants. Undiluted antisera were added to appropriate wells, and the plates were incubated in a moist chamber at 25° for 24-72 hr.

Protein blotting procedure.--Protein blotting was performed as described by Towbin et al. (1979) and Batteiger et al. (1982). Transfers were performed in transfer buffer [25 mM Tris; 192 mM glycine; 20% (v/v) methanol pH 6.5], at 4° for 3 hr at 1 A using a TE 42 transphore-electrophoretic transfer unit (Hoefer Scientific Instruments, San Francisco, California, 94107) and nitrocellulose membranes (NCM) (0.45 µm pore size; Schleider and Schull, Keen, NH). After transfer, NCM (70 X 140 mm) were sequentially subjected to 1) incubation with a blocking agent buffer [PBS containing 0.05% v/v polyoxyethylene sorbitan monolaureate (Tween-20)] at 37° for 1 hr; 2) incubation with 100 ml of 1:200 dilution of selected immune sera in PBS-Tween-20 buffer at 37° for 2 hr; 3) a brief wash at room temperature with PBS-Tween-20 buffer; 4) incubation with 0.25 µCi of ^{125}I-protein A diluted to 50 ml with PBS-Tween-20 buffer at 37° for 1 hr; and 5) two final washes at room temperature for 30 min, each with PBS-Tween-20 buffer. Processed NCM

were dried and exposed for autoradiography for 48 hr at -85°.

Microscopy.--The AI were examined *in situ* in epidermal strips of PeMV infected tobacco and PRSV-W infected zucchini squash leaves after clearing the cells by immersing tissue in 5% Triton X-100 for 3-5 min and staining according to methods described by Christie and Edwardson (1977). Healthy tissues were used as controls. The AI were monitored during purification by light microscopic examination of preparations stained with 0.15% aqueous Phloxine B (Christie, 1967) or with the luxol brilliant green BL-calcomine orange ZRS technique (Christie and Edwardson, 1977), hereafter referred as O-G stain. Infected leaf tissue was fixed, embedded and sectioned for electron microscopy (Spurr, 1969). The ultrathin sections were mounted on Formvar coated grids, post stained with uranyl acetate-lead citrate and examined with a Hitachi H-600 electron microscope. Semipurified AI which were examined by microscopy of ultrathin sections were prepared as described by Knuhtsen *et al*. (1974) for partially purified tobacco etch virus (TEV) nuclear inclusions. The intracellular location of AI was examined using AI protein antisera directly labeled with rhodamine isothiocyanate (Hapner and Hapner, 1978). Epidermal strips of healthy and infected tissue were treated according to Hiebert *et al* (in press a). Nonspecific binding of antibodies was minimized by 30 min preincubation of antisera (27 µl) with healthy tissue extract [27 µl of tissue extracted in 10 fold (weight) saline buffer] (N. Ko and E. Hiebert, unpublished data). Nonspecific binding of antibodies was tested by using preimmune sera with infected tissue and by exposing healthy tissue to anti-AI protein sera. Unstained sections were used to estimate the extent of autofluorescence from the tissue.

Results

Microscopy of amorphous inclusions.--Amorphous inclusions occurred consistently in tobacco and zucchini leaves infected with PeMV and PRSV-W, respectively, but they were never seen in healthy plants. When viewed with the light microscope, AI appeared to be imperfect spherical structures (Fig. 1). Usually one or two AI were found per cell, but not all cells contained them. Treatment with the O-G stain produced a green to olive brown coloration, indicating the presence of substantial amounts of protein (Figs. 1A, C), whereas Azure A stained AI red to deep violet, which indicated the presence of nucleic acid (Figs. 1B, D). Electron microscopy of PRSV-W infected tissue revealed the presence of AI consisting of a uniformly dense matrix with small electron-translucent gaps (Fig. 2B). The AI observed by electron microscopy of thin sections of PeMV infected tobacco tissue, however, revealed that their matrix was not as uniformly electron-opaque as that of the AI in PRSV-W infected zucchini squash tissue. The electron-translucent gaps were larger and more abundant in the case of PeMV, producing a less compact structure (Fig. 2A).

Purification.--Shape, size and density of AI were important features in developing the isolation procedure. The AI can be readily seen and differentiated from cellular components by light microscopy using selective stains (Figs. 1A, B, C, D) and therefore this technique was used to monitor the progress of isolation. The AI are so large that they will readily sediment through viscous sucrose solutions at relatively low centrifugal forces. This property greatly simplified the purification procedure.

Figure 1. Light micrographs of infected epidermal tissues showing amorphous inclusions *in situ*. *Nicotiana tabacum* var. NN infected with PeMV, stained with the O-G stain (A), and with Azure-A (B). *Cucurbita pepo* var. Chefini infected with PRSV-W stained with the O-G stain (C) and with Azure-A (D). AI, amorphous inclusion; CI, cylindrical inclusion.

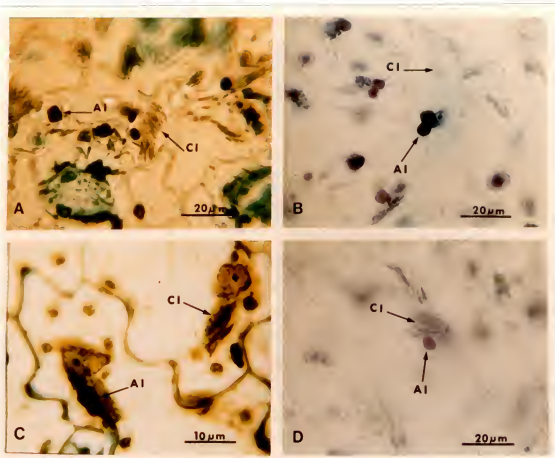
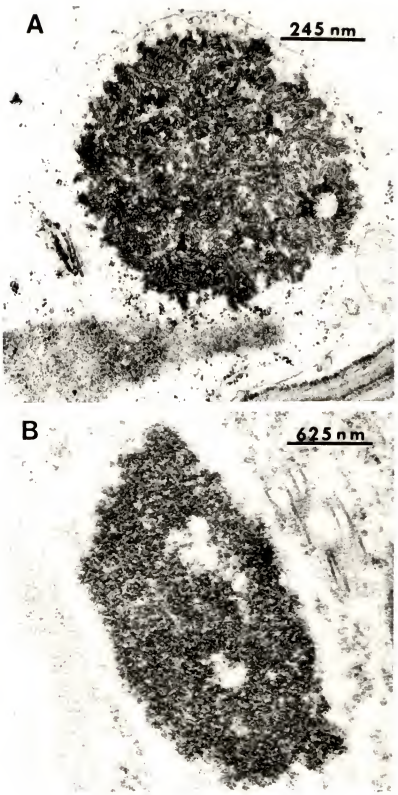

A
AI
CI
20μm
B
CI
AI
20μm
C
CI
AI
10μm
D
CI
AI
20μm

Figure 2. Electron micrographs of infected tissues showing amorphous inclusions *in situ*. *Nicotiana tabacum* var. NN infected with PeMV (A), and *Cucurbita pepo* var. Chefini infected with PRSV-W (B).


A
245 nm
B
625 nm

Comparisons by light and electron microscopy of semipurified AI (Figs. 3A, B; Figs. 3C, D) to those seen in situ (Figs. 1A, B, C, D; Figs. 2A, B) indicated that the semipurified AI had retained their characteristic structures. The pattern of electrophoretic migration of semipurified, SDS degraded AI protein, is shown in Fig. 4. The major protein component of semipurified AI migrated with apparent Mr 51k for both PeMV and PRSV-W. These proteins were absent from extracts of healthy plants treated in the same manner. The homogeneity of SDS-PAGE purified preparations was confirmed by the presence of a single band in a second SDS-PAGE (data not shown). Absorbance produced by purified AI proteins degraded in SDS ranged up to 35 (PRSV-W) and 55 (PeMV) A_{280} units per kg of infected tissue.

Antisera and SDS immunodiffusion tests.--Reactive AI protein antisera were obtained two weeks after the final injection of the rabbits. The AI antigens in infected plant extracts gave prominent reactions when tested against AI protein antisera (Fig. 5). None of the antisera reacted with sap from healthy plants.

Binding of proteins to NCM.--The specificity of AI antisera was tested by Western blot analysis. Proteins in the semipurified AI preparation were separated by SDS-PAGE. The separated proteins were electrotransferred to NCM and tested for serological activity. The results indicated a specific and unique recognition of AI protein by AI protein antisera (Figs. 6, 7). The Mr 51k AI proteins were serologically distinct from either the coat or cylindrical inclusion proteins. No binding of normal rabbit immunoglobulins was observed (Figs. 6, 7)

Specificity of the immunofluorescent staining procedure.--The reactivities of AI antisera conjugated with rhodamine were tested against

Figure 3. Photomicrographs of extracted amorphous inclusions of PeMV (A) stained with 0.15% Phloxine-B and PRSV-W (B), stained with the O-G combination. Electron micrograph of thin sections through embedded pellets of semipurified amorphous inclusions induced by PeMV (C) and by PRSV-W (D). AI, amorphous inclusion; CI, cylindrical inclusion.

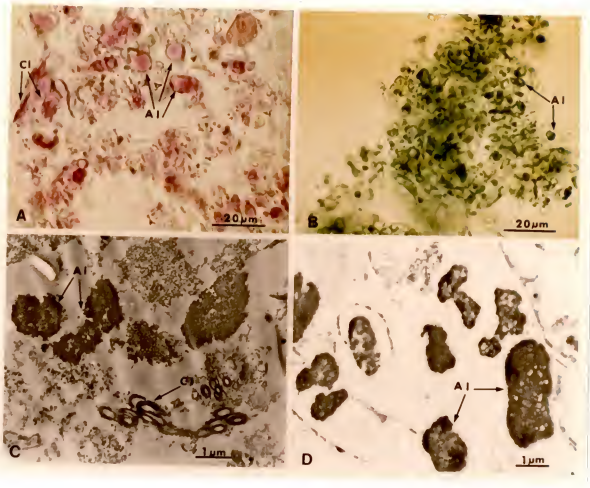

Cl
Al
A
20μm
Al
B
20μm
Al
Cl
C
1μm
Al
D
1μm

Figure 4. Polyacrylamide gel electrophoresis of sodium dodecyl sulfate (SDS) dissociated proteins in PeMV and PRSV-W semipurified amorphous inclusion preparations. I, extract from infected tissues; HT, extract from healthy tobacco tissue; HS, extract from healthy squash tissue; M, marker proteins (in Kilodaltons). Marker proteins are: tobacco mosaic viral coat protein subunit (17.5k); carbonic anhydrase (29k); glutamate dehydrogenase (53k); bovine serum albumin (67k); phosphorylase b (94k) and myosin (200k). AI protein mol. wts. (51k) indicated by arrows.

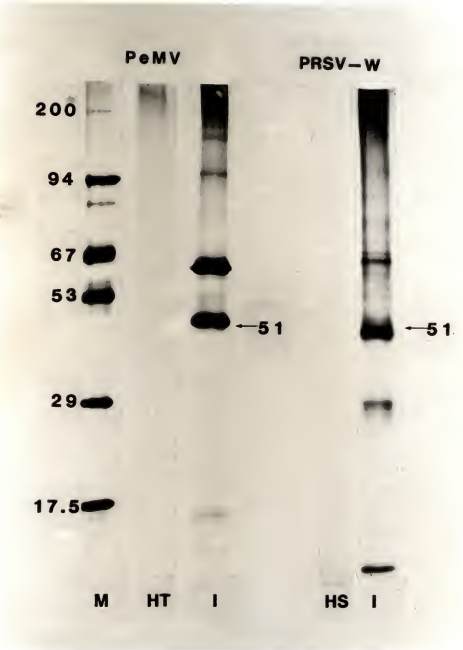

PeMV
PRSV-W
200
94
67
53
29
17.5
51
51
M
HT
I
HS
I

Figure 5. Serologic detection of amorphous inclusion protein in sodium dodecyl sulfate (SDS)-immunodiffusion tests. Central wells contain A, PeMV AI protein antiserum; B, PRSV-W AI protein antiserum. The peripheral wells contain SDS-treated sap from: P, PeMV infected tobacco plants; W, PRSV-W infected zucchini plants, and H, healthy plants.


P
H
P
A
P
H
P


W
H
W
B
W
H
W

Figure 6. Western blot analysis of the proteins contained in semipurified PeMV amorphous inclusion preparations. After protein transfer, nitrocellulose membranes were incubated with lane 1, PeMV AI protein antiserum; lane 2, PeMV capsid protein antiserum; lane 3, PeMV cylindrical inclusion protein antiserum; lane 4, normal serum. Resulting antigen-antibody complexes [mol. wts. in Kilodaltons (K), indicated by arrows] were visualized by autoradiography after incubation with [^{125}I] protein A (0.25 µCi) (exposure 48 hr).

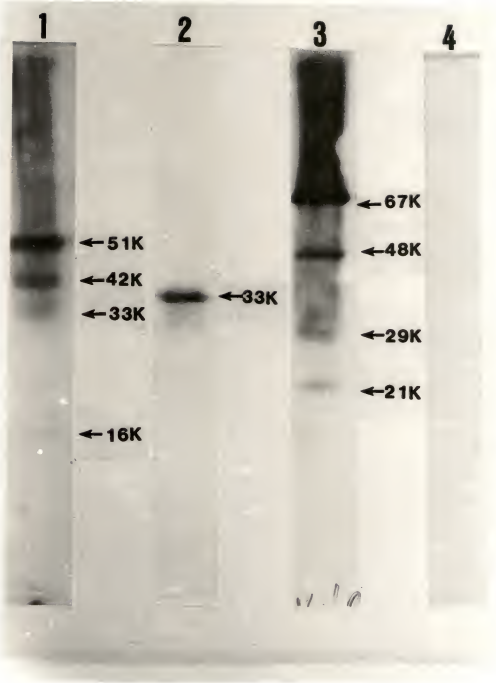
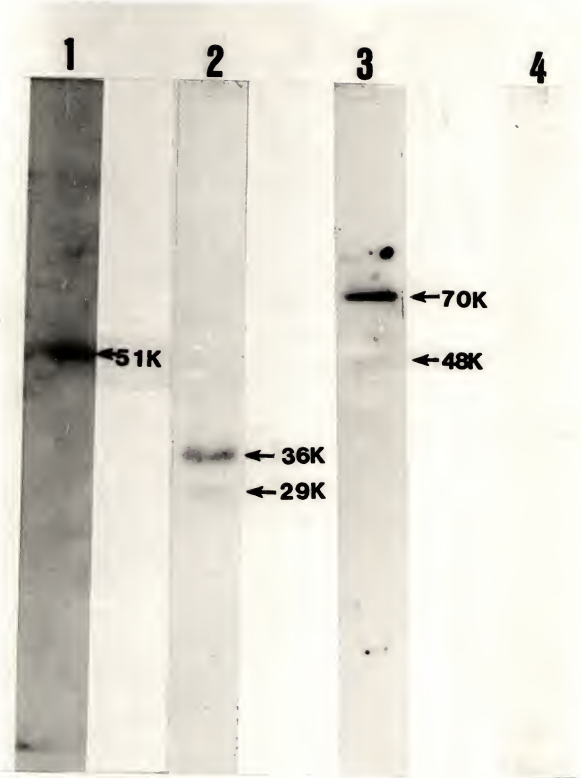

1
2
3
4
←51K
←42K
←33K
←16K
←33K
←67K
←48K
←29K
←21K

Figure 7. Western blot analysis of the proteins contained in semipurified PRSV-W amorphous inclusions preparations. After protein transfer, nitrocellulose membranes were incubated with lane 1, PRSV-W AI protein antiserum; lane 2, PRSV-W capsid protein antiserum; lane 3, PRSV-W cylindrical inclusion protein antiserum; lane 4, normal serum. Resulting antigen-antibody complexes [mol. wts. in Kilodaltons (K), indicated by arrows] were visualized by autoradiography after incubation with [^{125}I] protein A (0.25 µCi) (exposure 48 hr).

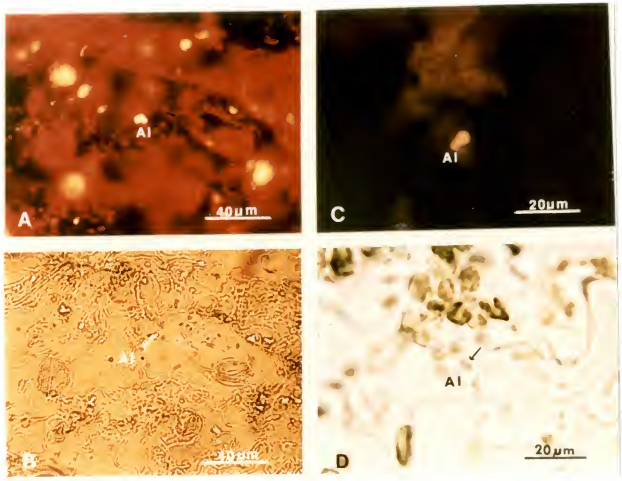

1
2
3
4
51K
36K
29K
70K
48K

the AI *in situ*. In Figs. 8B and D, epidermal strips photographed with visible light show the intracellular location of AI. In Figs. 8A and C, the same field of view shows the specific immunofluorescence of AI in tissue treated with rhodamine-labeled AI antiserum.

Discussion

Isolation and purification of large amounts of the AI of PeMV and PRSV-W were made possible by the fact that the inclusions were quite stable and remained essentially intact and recognizable during the procedure. The physical appearance of the AI as revealed by light microscopy was the only criterion I had to evaluate whether or not the AI had been denatured. The use of either the O-G combination or the Phloxine B stain was a quick and useful approach for monitoring the progress of purification. The AI were readily detected by their staining reaction and their peculiar shape, contrasting with the unstained starch grains, some cellular components and unidentified host debris (Figs. 3A, B). The electron microscopic examination of thin sections through pellets of semi-purified AI preparations revealed the presence of similar inclusions to those observed with light microscopy (Figs. 3C, D). However, these inclusions did not show the evident substructure seen in the AI in ultrathin sections of infected tissue (Fig. 2); it is likely that the treatments used for purification, fixing, embedding and staining the isolated AI modified their ultrastructural appearance. Cylindrical inclusions were seen in light as well as in electron microscopic observations of semi-purified PeMV AI preparations (Figs. 3A, C). These inclusions are fragments of the large cylindrical inclusions that were broken apart during the purification. No cylindrical inclusions were observed in PRSV-W AI pellets (Figs. 3B, D).

Figure 8. Localization of amorphous inclusions by immunofluorescence microscopy. A) PeMV-infected tobacco epidermal strip photographed with epifluorescence optics showing the specific fluorescence from AI treated with rhodamine labeled antiserum to PeMV AI protein. B) the same field of view photographed with visible light. C) PRSV-W infected zucchini epidermal strip photographed with epifluorescence optics, showing the specific fluorescence from AI treated with rhodamine labeled antiserum to PRSV-W AI protein. D) the same field of view photographed with visible light. AI, amorphous inclusion. Arrows indicate AI.


A
Al
40µm
C
Al
20µm
B
Al
D
Al
20µm

The primary constituent of the purified AI was a Mr 51k protein which was serologically distinct from viral capsid, cylindrical inclusion and host protein as was shown by the electrophoretic blotting technique used here. Proteins yields, based on A_{280} of the purified preparations, suggest that significant quantities of serologically unique, nonstructural protein are produced and assembled into AI in PeMV and PRSV-W infected tissue. Although SDS-treated AI proteins were used as immunogens, the resulting antisera were able to recognize antigenic sites on the nondenaturated AI *in situ* (Fig. 8). The antigenicities of the AI proteins were preserved, at least in part, despite the presumed denaturing effect of the SDS-PAGE. Similar results have been observed with antisera prepared to potyviral CI and TEV NI (E. Hiebert, unpublished data). The existence of specific sera directed against the major component of the AI induced by these two potyviruses should be useful probes for studying the time course of appearance of AI during the infection cycle in infected tissues and protoplasts. The function and physiological significance of the PeMV- and PRSV-W AI are unknown, but evidence will be presented in Chapter 2 that the AI proteins, like the cylindrical inclusion and nuclear inclusion proteins, are of viral origin.

CHAPTER 3

IDENTIFICATION OF POTYVIRAL AMORPHOUS INCLUSION PROTEIN AS A NONSTRUCTURAL, VIRUS-SPECIFIC PRODUCT RELATED TO HELPER COMPONENT.

Introduction

The viral origin of nonstructural proteins associated with potyviral cylindrical inclusions, nuclear inclusions and helper component (HC) is a well documented fact, and most of the genes coding for these nonstructural proteins have been mapped on the potyviral genome (Dougherty and Hiebert, 1980b; 1980c; Hellman et al., 1983; Hiebert, 1981; Hiebert et al, in press b; Purcifull et al., in press). The potyviral genetic map proposed by Dougherty and Hiebert (1980c) illustrated the genetic potential for the production of additional nonstructural proteins in potyviral infections. The preparation of antisera to the amorphous inclusions (AI) associated with infections caused by pepper mottle virus (PeMV) and a watermelon mosaic virus-1 strain of papaya ringspot virus (PRSV-W) (Purcifull et al., in press) (Chapter 1) presented an opportunity to identify and map additional potyviral-specified proteins.

In this chapter, antisera to the PeMV and PRSV-W AI proteins were used to identify unique gene products synthesized by PeMV and PRSV-W genomes in a rabbit reticulocyte lysate (Dougherty and Hiebert, 1980a). The major in vitro translation products of PeMV and PRSV-W RNAs reacted with antisera to their respective AI proteins and generated proteolytic

peptides similar to those generated by the respective purified AI proteins. Thus, the constituent protein associated with PeMV and PRSV-W AI represents another potyviral-coded nonstructural protein.

Materials and Methods

Virus source and propagation.--PeMV and PRSV-W source and propagation have been described in Chapter 1.

Virus purification.--PeMV was purified according to the method published by Dougherty and Hiebert (1980a).

The PRSV-W isolate was purified from infected zucchini squash plants. Leaf tissue (100 g), collected 2-3 weeks after inoculation, was homogenized in a Waring blender for 2 min at 4° in 200 ml of solution containing 500 mM potassium phosphate buffer, pH 7.5 (PB), 0.1% sodium sulfite (w/v), 0.5 ml chloroform and 0.5 ml carbon tetrachloride per gram of tissue. The homogenate was expressed through cheesecloth and the filtrate was centrifuged at 1020 g for 5 min. The pellet was reextracted with 100 ml PB and centrifuged again. Both supernatants were combined, centrifuged at 13,000 g for 15 min and the pellet was discarded. The supernatant was clarified by the addition of Triton X-100 to a final concentration of 1%, and the virus was precipitated from the supernatant by adding polyethylene glycol (PEG Mr 8000) to a final concentration of 4% (w/v) and 100 mM NaCl while stirring for 1 hr at 4°. The precipitated virus was collected by centrifugation at 10,400 g for 10 min. The resulting pellet was resuspended in 50 ml of 20 mM of PB with the aid of a glass tissue grinder. The resuspended pellet was centrifuged at 12,000 g for 10 min and the pellet was discarded. The virions were reprecipitated by stirring the supernatant with PEB to a final concentration of 8% (w/v) and 100 mM NaCl for 30 min at 4°. The

precipitated virus was centrifugated at 12,000 g for 10 min and the pellet was resuspended in 5-10 ml of 20 mM PB with the aid of a glass tissue grinder. The resuspended pellet was subjected to centrifugation on a CsCl gradient (30% CsCl in 20 mM PB generated) at 14,000 g for 18 hr at 5°. The virus zone was removed and diluted with an equal volume of 20 mM PB and then centrifuged at 12,000 g for 10 min. The virus was removed from the CsCl in the supernatant by PEG precipitation. Virus yields were in the range of 5-10 mg/100 g tissue.

RNA isolation. RNAs were extracted by dissociating freshly purified virus in an equal volume of 200 mM ammonium carbonate (pH 9.0), 2 mM ethylene diamine tetracetate (EDTA), and 2% sodium dodecyl sulfate (SDS) (Brakke and Van Pelt, 1970a) and isolated by rate zonal density gradient centrifugation in linear-log sucrose gradients (Brakke and Van Pelt, 1970b). The 39 S RNA fraction was collected and precipitated by the addition of sodium acetate (pH 5.0, final concentration 100 mM) and two volumes of 100% ethanol. The RNA was resuspended in a small volume of H_2O and frozen at -85° until used for translation.

Lysate preparation and in vitro translation conditions.--The in vitro translation conditions and procedures were as described previously (Dougherty and Hiebert, 1980a). Rabbit reticulocyte lysate was obtained from Green Hectares, Oregon, Wisconsin 53575. The antisera used were PeMV coat protein (Purcifull et al., 1975); PeMV CI protein (Batchelor, 1974); PeMV AI protein (Chapter 1); PRSV-W coat protein (Purcifull and Hiebert, 1979); PRSV-W CI protein (Hiebert and Purcifull, unpublished data); PRSV-W AI protein (Chapter 1); TEV NI proteins (Dougherty and Hiebert, 1980b); and TVMV HC protein (Thornbury and Pirone, 1983). Partial proteolytic digestion of the in vitro synthesized products,

immunoprecipitable with antisera to AI proteins, was performed using Staphylococcus aureus V-8 protease as described by Cleveland et al. (1977).

Protein blotting procedure.--Electrotransfer and protein blotting procedures have been described in Chapter 1.

Results

Immunological recognition of in vitro translated products and peptide mapping of in vitro translated products and purified amorphous inclusion proteins.--The in vitro translated products of both PeMV and PRSV-W RNAs were analyzed by electrophoresis in SDS discontinuous polyacrylamide gradient gels. Translation of each viral genome resulted in unique products under identical conditions (Fig. 9; Fig. 10). The major product of PeMV translation had an estimated molecular weight (Mr) of 78,000 (78k), whereas the major product of PRSV-W translation had an estimated Mr of 110k. The in vitro translation products were examined with the use of a variety of antisera prepared against potyvirus-specific proteins. When translation products were treated with the respective AI protein antisera the Mr 78k product from PeMV and the Mr 110k product from PRSV-W RNAs were immunoprecipitated. A minor product of Mr 49k from PeMV and of Mr 47k from PRSV-W were also immunoprecipitated. The high molecular weight products (78k and 110k) were not immunoprecipitated by cylindrical inclusion (CI) antisera, by capsid antisera, or by tobacco etch virus (TEV) nuclear inclusion (NI) protein antisera. No polypeptides were detected by immunoprecipitation when the cell-free products were tested with normal serum.

The peptide S. aureus V-8 protease digest patterns of the purified AI proteins were compared with the digest patterns of the translation

Figure 9. Analysis of the *in vitro* translation products of PeMV RNA by immunoprecipitation with antiserum prepared to PeMV amorphous inclusion (AI) protein. The figure illustrates [^{35}S] methionine-labeled products separated in a sodium dodecyl sulfate (SDS) polyacrylamide slab gel (7.5% to 15%) gradient (PAGE) and detected by fluorography. Lane 1, [^{14}C] marker proteins. Molecular weight markers in Kilodaltons are from the bottom to the top: carbonic anydrase (29k); ovalbumin (46k); bovine serum albumin (67k); phosphorylase b (92.5k) and myosin (200k). Lane 2, total products of PeMV RNA translation; lane 3, products immunoprecipitated with antiserum to PeMV AI protein; lane 4, products immunoprecipitated with antiserum to PeMV capsid protein; lane 5, products immunoprecipitated with antiserum to PeMV CI proteins; lane 6, products immunoprecipitated with antiserum to TEV-54k nuclear inclusion protein; lane 7, products immunoprecipitated with antiserum to TEV-49k nuclear inclusion protein; and lane 8, products immunoprecipitated with normal serum. The numbers at the left refer to the molecular weights of the PeMV-RNA total products.

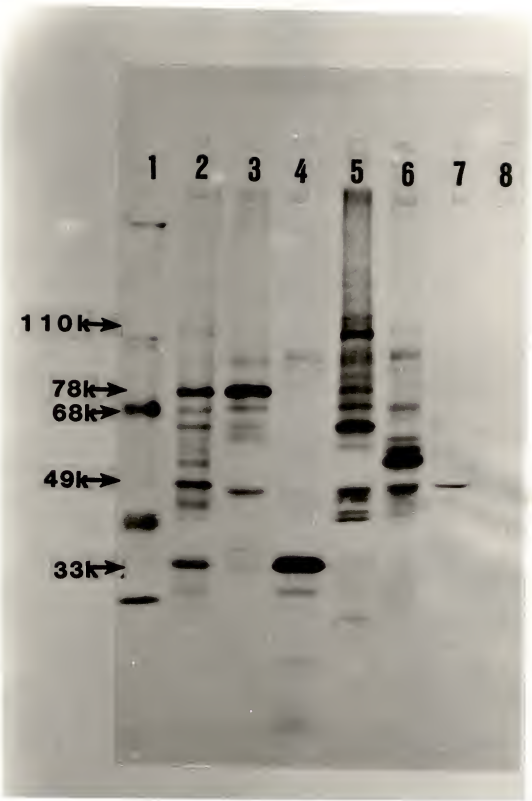
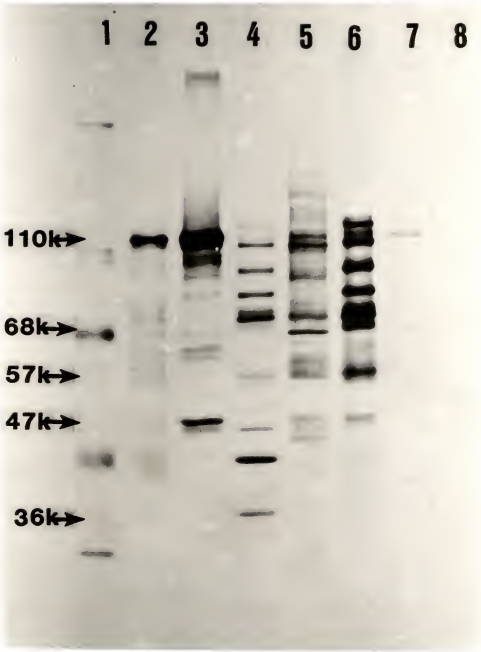

1
2
3
4
5
6
7
8
110k
78k
68k
49k
33k

Figure 10. Analysis of the in vitro translation products of PRSV-W RNA by immunoprecipitation with antisera prepared to PRSV-W amorphous inclusions (AI) protein. The figure illustrated [^{34}S] methionine-labeled products separated in a sodium dodecyl sulfate (SDS)-polyacrylamide slab gel (7.5% to 15%) gradient (PAGE) and detected by fluorography. Lane 1, [^{14}C] marker protein. Molecular weight markers in Kilodaltons are from the bottom to the top: carbonic anydrase (29k); ovalbumin (46k); bovine serum albumin (67k); phosphorylase b (92.5k) and myosin (200). Lane 2, total products of PRSV-W RNA translation; lane 3, products immunoprecipitated with antiserum the PRSV-W AI protein; lane 4, products immunoprecipitated with antiserum to PRSV-W capsid protein; lane 5, products immunoprecipitated with antiserum to PRSV-W cylindrical inclusion protein; lane 6, products immunoprecipitated with antiserum to TEV-54k nuclear inclusion protein; lane 7, products immunoprecipitated with antiserum to TEV-49k nuclear inclusion protein; and lane 8, products immunoprecipitated with normal serum. The numbers at the left refer to the molecular weights of the PRSV-W RNA total products.


1
2
3
4
5
6
7
8
110k
68k
57k
47k
36k

products immunoprecipitated with antisera against AI proteins (Fig. 11). The polyacrylamide gel electrophoresis (PAGE) digest pattern of the [^{35}S] methionine labeled translation products revealed similar peptides to those generated by purified AI proteins stained with silver stain.

Western blot analysis.--When proteins in the semipurified PRSV-W AI preparation (Chapter 1) were electrotransfered to nitrocellulose membranes and tested with TVMV-HC protein antiserum (Fig. 12, lane 4) the immunoreactive product had the same molecular weight as the product immunoreactive with PRSV-W AI protein antiserum (Fig. 12, lane 1). The immunoreactive product was different from coat or cylindrical inclusion proteins (Figs. 12, lanes 2 and 3). No binding of normal immunoglobulins was observed (Fig. 12, lane 5).

Discussion

The immunoprecipitation analyses of the *in vitro* translation of PeMV and PRSV-W genomes, using the AI protein antisera, provided direct evidence that the AI proteins are potyviral-coded protein. The comparison of the proteolytical peptides generated by the translation products immunoprecipitated by the AI antisera with those generated by the authentic AI proteins verified the relationship of the *in vitro* products to the *in vivo* proteins. However, the SDS-PAGE analyses (Figs. 9 and 10) of the translation products immunoprecipitated with the AI antisera did not show products comigrating with PeMV or PRSV-W AI proteins.

Three hypotheses may be proposed to account for the absence of products comigrating with the Mr 51k AI protein in the *in vitro* translation analyses. First, the Mr 78k product from PeMV and the Mr 110k from PRSV-W RNAs may represent proteins from incomplete proteolytic

Figure 11. Staphylococcus aureus V-8 protease digest pattern comparison of the in vivo amorphous inclusion (AI) protein with the in vitro translation products immunoprecipitated with antisera to AI proteins. The protease products were separated by SDS-PAGE. In A, lanes 1-3 are silver-stained digest patterns of the in vivo PeMV AI protein; lanes 4-6 are fluorograms of the digest pattern of PeMV RNA translation products immunoprecipitated by PeMV AI antiserum. In B, a similar comparison of labeled PRSV-W RNA translation products (lanes 4-5) and stained PRSV-W AI protein (lanes 1-2). The products of translation and the AI proteins are identified by their estimated molecular weights. Arrows indicate the common peptides. Digestion times were 15 minutes: 1 and 4 (A) (B); 30 minutes: 2 and 5 (A) (B); 60 minutes: 3 and 6 (A). Asterisks indicate components of the V-8 protease preparation consistently observed in silver stained gels.

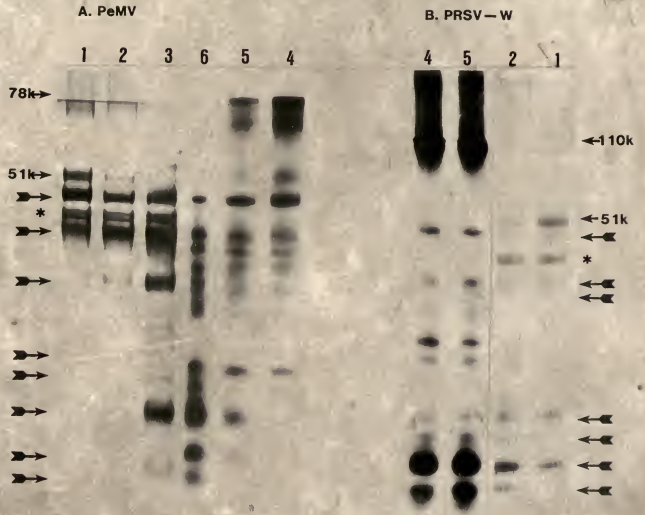
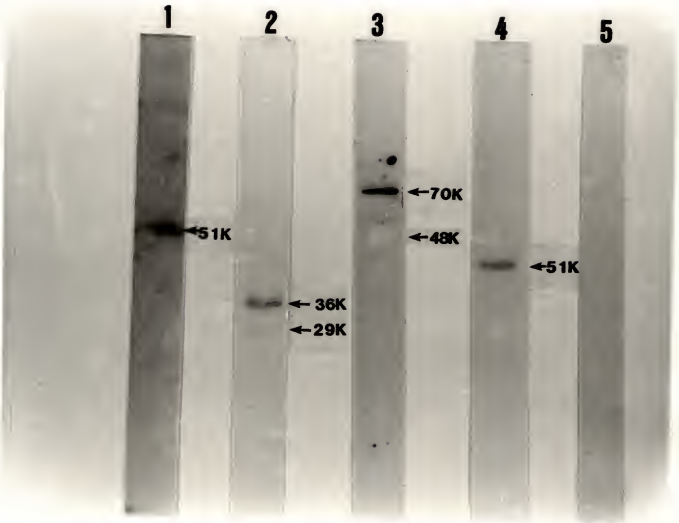

A. PeMV
1 2 3 6 5 4
78k
51k
*
B. PRSV—W
4 5 2 1
110k
51k
*

Figure 12. Western blot analysis of the proteins contained in semipurified PRSV-W amorphous inclusion (AI) preparations. After protein transfer, nitrocellulose membranes were incubated with: lane 1, PRSV-W AI protein antiserum; lane 2, PRSV-W capsid protein antiserum; lane 3, PRSV-W cylindrical inclusion protein antiserum; lane 4, tobacco vein mottling virus helper component (TVMV HC) antiserum; lane 5, normal serum. Resulting antigen-antibody complexes [mol. wts. in Kilodaltons (K), indicated by arrows] were visualized by autoradiography after incubation with [^{125}I] protein A (0.25 µCi) (exposure 48 hr).


1
2
3
4
5
51K
36K
29K
70K
48K
51K

processing of a polyprotein. This hypothesis involves specific proteolytic processing of a large polypeptide formed by translation beginning at a single initiation site and proceeding through the entire length of the genome. The ultimate products are formed by specific proteolytic cleavages of the larger polypeptide precursor, as reported in the translations of cowpea mosaic virus (Pelham, 1979; Goldbach et al., 1980, 1981, 1982; Goldbach and Rezelman, 1983). Secondly, when viral RNA is translated in a cell-free system, new initiation and termination sites could be produced by RNA degradation (Pelham, 1979). This could result in the synthesis of polypeptides differing in size but containing the amino acid sequences of virus specified proteins produced in vivo. In this case, the expression of potyviral genome in vitro may not involve authentic initiation or termination codons. The third hypothesis considers that the immunoprecipitations of the Mr 78k and 110k products by the Mr 51k AI protein antisera may represent a readthrough of two genes at the 5' end of the PeMV and PRSV-W RNAs. When TEV-RNA is translated in the wheat germ system a major product of Mr 36k is produced. The Mr 36k product is unreactive with tobacco vein mottling virus (TVMV) helper component (HC) protein antiserum which reacts with the major Mr 87k product of TEV-RNA translated in the rabbit reticulocyte lysate system (Hiebert et al., in press b; Hiebert, unpublished data). These two products (36k and 87k) may have overlapping sequences and thus be representative of two genes instead of just one at the RNA 5' end as is proposed in the map of Dougherty and Hiebert (1980c). If this is true, the initiation codon for the AI gene may be downstream (toward the RNA 3' end) from the initiation codon for the Mr 78k and 110k products. In fact, the immunoprecipitations and peptide mappings with products

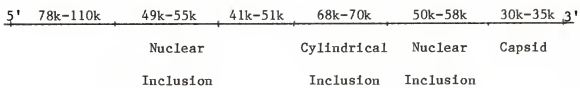
translated in the reticulocyte lysate system indicated that PeMV and PRSV-W AIs sequences were congruent with a part of the sequences of the Mr 78k and 110k products, respectively. The Mr 78k and 110k products may, indeed, represent readthrough products. Confirmation of whether the Mr 36k product from TEV RNA, as translated in the wheat germ system, is related to the major Mr 87k product of TEV RNA translation in the rabbit retyculocyte lysate system needs to be determined by peptide mapping.

The Mr 51k protein gene has been mapped near the 5' end of the potyviral genome (Fig. 13) on the basis of the immunoprecipitation analyses of the in vitro translation products. Antisera to the Mr 51k AI proteins reacted specifically with the major translation product of PeMV and PRSV-W RNAs. The major translation product of the potyviral genome has been mapped at the 5' end (Dougherty and Hiebert, 1980c). The proposed Mr 51k AI protein gene may be located downstream within the primary in vitro translation product gene (Fig. 13) for reasons discussed in the third hypothesis above.

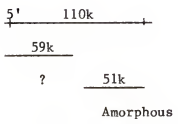
In preceding immunological analyses, potato virus Y (PVY) and TVMV-HC protein antisera were used to immunoprecipitate cell-free translation products of various potyviruses (Hiebert et al., in press b). TVMV HC protein antiserum efficiently immunoprecipitated the major translation product of PRSV-W RNA. The correlation between the immunoreactivities of the Mr 110k PRSV-W RNA in vitro translated product and the Mr 51k in vivo protein by both antisera (PRSV-W AI protein and TVMV-HC protein antisera) (Fig. 12, lane 1 and 4) opens a question about the possible similarity of the two proteins. Their serological affinities, expressed by the heterologous immunoreactivities, suggest that the immunoreactive products from PRSV-RNA contain a part, if not all, of the protein making up the HC

Figure 13. A proposed location of amorphous inclusion (AI) protein gene on the potyviral genome. The figure includes: A) the proposed gene order of all identified potyviral specified proteins (Hiebert, 1981); B) PRSV-W RNA and C) PeMV RNA, 5' end indicating the proposed location of the gene for AI protein. The molecular weights of the products identified by immunoprecipitation studies using various potyviral protein antisera are illustrated in A. In B and C the 51k AI protein gene of PRSV-W and PeMV, respectively, is located upstream within the primary product gene illustrated in A.

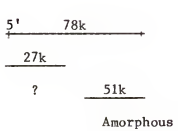
A.


5'
78k-110k
49k-55k
41k-51k
68k-70k
50k-58k
30k-35k
3'
Nuclear
Inclusion
Cylindrical
Inclusion
Nuclear
Inclusion
Capsid

B.


5'
110k
59k
?
51k
Amorphous
Inclusion

C.


5'
78k
27k
?
51k
Amorphous
Inclusion

protein in PRSV-W infected tissues. The PRSV-W Mr 51k protein has been identified *in vivo* as an AI constituent (Chapter 1). The HC protein of TVMV has been isolated as a soluble protein having an estimated Mr of 52k (Thornbury and Pirone, 1983). However, TVMV induces the formation of conspicuous fibrous cytoplasmic inclusions [Edwardson, 1974 (Fig. 10b, p. 301)] which may be related to the TVMV HC protein. These inclusions, as well as the AI, may represent a pool or a reservoir of the HC protein. Further experimentation will be required to answer the question about whether the HC and the AI protein are identical proteins. Such experiments will involve testing the possible helper activity of the AI protein and possible immunoinhibition of the aphid transmission by the AI protein antiserum. Regardless of the relationships between the HC and AI proteins, the data presented here are definitive evidence that the genetic material coding for the protein accumulated in the AI associated with PEMV and PRSV-W infections is contained in the potyviral genome.

CHAPTER 4

CONCLUSIONS

In this study I have described a method for the isolation of the amorphous inclusions (AI) associated with pepper mottle virus (PeMV) and papaya ringspot virus type W (PRSV-W) infections. Since the AIs induced by these two potyviruses were quite stable to physical and detergent treatments, the purification of these inclusions did not destroy the inclusion characteristic shape. The purification procedure outlined in this study may have application for the purification of other potyviral AI or other viral inclusions such as the tobacco mosaic virus X-bodies.

Semipurified PeMV and, PRSV-W AI preparations contained intact AI and, therefore, polyacrylamide gel electrophoresis (PAGE) of sodium dodecyl sulfate (SDS) dissociated AI suggest that inclusions are made of a single kind of protein subunit with a molecular weight (Mr) of 51,000 (51k). The cylindrical inclusions (CI) induced by all potyviruses are also complex structures made up entirely of a single protein subunit type (Hiebert and McDonald, 1973). However, SDS-PAGE of tobacco etch virus (TEV) nuclear inclusion (NI) revealed a two protein composition (Knuhtsen et al., 1974). The AIs induced by PeMV and PRSV-W are similar in shape, composition and stain reaction. A study of relationships needs further experimentation, such as peptide mapping of the proteins present in isolated AIs, reciprocal immunological testing of the resulting proteolytical peptides by Western blot and protein isoelectrofocusing.

Tobacco vein mottling virus (TVMV) helper component (HC) protein antiserum, used as a new probe for studying potyviral genome relationships, has been able to immunoprecipitate a unique subset of *in vitro* translated products from a considerable number of potyviral RNAs (Hiebert *et al.*, in press b). The polypeptides precipitated by TVMV-HC antiserum were immunoprecipitated by AI protein antiserum but not by other potyviral protein antisera. Thus it is clear that these antisera define a new cistron in the potyviral genome. TVMV-HC protein and PRSV-W AI protein have almost the same Mr (51-52k), and are serologically cross-reactive. The size of these proteins as well as their serological relationship has been confirmed in *in vivo* and *in vitro* studies. The HC and the AI are similar and they may be identical proteins. Based on these observations, it is reasonable to expect some degree of cross relationships between PeMV and PRSV-W AI protein as has been observed in this study. Perhaps the AI represent a reservoir of HC for PeMV. When ongoing studies about the helper activities of PeMV and PRSV-W AI proteins are concluded this question may be answered.

In vitro translation studies with potyviral RNAs (Dougherty and Hiebert, 1980a, b; Hellman *et al.*, 1980, 1983) have established the existence of at least six gene products. The *in vivo* synthesis of five of these products have now been verified in potyviral infections. Four of the products are accumulated in inclusions. Based on immunoprecipitation of *in vitro* translated products, a genetic map for the potyviral genome has been proposed by Dougherty and Hiebert (1980c). The proposed gene order is 5'-p78-110-p49-p41-50-p67-70-p54-56-p30-36-3'. The characterization of the AI protein, and its identification as a virus coded protein offered the opportunity to

analyze the gene products coded for by the PeMV and PRSV-W RNAs 5' end in greater detail. This analysis suggests the resolution of two genes (Fig. 13 B and C) instead of one (Fig. 13 A) near the 5' terminus of potyviral RNAs. Now the proposed genetic map is as follows: 5'-p30-60-p51-56-p49-p41-50-p67-70-p54-56-p30-36-3'.

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BIOGRAPHICAL SKETCH

Maria Victoria Garagorry de Mejia was born in Montevideo, Uruguay, on May 20, 1942, and became a Venezuelan citizen in 1963. She attended the Universidad de Oriente, Cumana, Venezuela, receiving the Licenciado en Biologia degree in 1973. In September 1974 she was hired as an Instructor Professor of Biology by the Universidad de Oriente. She attended the Instituto Venezolano de Investigaciones Cientificas (IVIC) from February 1975 to October 1977, earning her Magister Scienciarum degree in virology. In July 1977 she was promoted to Assistant Professor, a position that she has held up to the present time. In January, 1981, she entered the University of Florida, and expects to receive the degree of Doctor of Philosophy in April, 1984. Maria V. G. de Mejia is married to Dr. Pedro R. Mejia and has three children: Laura, Maria Alexandra and Pedro Julio. She is a member of the American Phytopathological Society, the Venezuelan Asociation for Science Advance (ASOVAC), the Association of University Professors from Universidad de Oriente (APUDO) and the Universidad de Oriente Alumni Association.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Dan E. Purcifull

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This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School, and was accepted as partial fulfillment of the requirements for the degree of Master of Science.

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